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Organization and Regeneration Ability of Spontaneous Supernumerary Eyes in Planarians —Eye Regeneration Field and Pathway Selection by Optic Nerves—

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ABSTRACT—Planarians can propagate asexually by fission and successive regeneration. During head regeneration, they again form a new pair of eyes, and sometimes supernumerary eyes. The positions of normal and supernumerary eyes and their regeneration abilities are expected to be highly relevant to the question of where and how the field to regenerate eyes is determined. In this study, spontaneously generated supernumerary eyes were classified into various types. In all cases, they were formed in the anterior part of the head. Enucleation of a normal eye elicited regeneration of a new eye; however, enucleation of a supernumerary eye did not. The supernumerary eyes were morphologically and functionally indistinguishable from the normal eyes, revealed by the studies of immunohistology and photophobic response, respectively. From the obtained results, we proposed a model of the eye regeneration field that changes its distribution spatiotemporally during regeneration. Immunohistological studies also showed that the optic nerves from the normal and supernumerary eyes ran independently, which might have implication about the nature of guidance cues for the optic nerves.

INTRODUCTION

Planarians of the common Japanese species *Dugesia japonica* propagate both sexually and asexually. Their asexual reproduction is initiated by fission and followed by regeneration: the anterior piece regenerates a tail, and the posterior piece regenerates a head. Regeneration of a head with a pair of eyes is also observed a few days after artificial decapitation. Moreover, an eye in the head region is regenerated readily after enucleation. As animals cannot usually regenerate the sensory-nervous system, the strong regeneration ability of planarians is very intriguing. What mechanisms are at work in planarian eye regeneration?

The planarian eye is composed of pigment cells and photoreceptors (Kishida, 1967; Kuchiiwa *et al*., 1991). The pigment cells form a pigment eye cup, while the photoreceptors are located outside of the pigment eye cup and have two types of processes. One type of process enters the pigment eye cup and forms rhabdomeres, an assembly of microvilli which may be associated with photopigments (Azuma *et al*., 1994, 1999). The other processes are optic nerve fibers projecting onto the brain. A monoclonal antibody (MA-VC1) specific for planarian photoreceptors was produced and used to determine the structure of the photoreceptors (Agata *et al*., 1998). Those authors described the optic pathway, including chiasma, and the relation between the optic nerve termination and the brain structure.

Two symmetrical eyes are usually regenerated after fission or decapitation, but supernumerary eyes are sometimes formed. When planarians are kept in a crowded state, the rate of occurrence of supernumerary eyes in the regenerating heads is invariably increased (Kanatani, 1957a). The effect of crowding on supernumerary eye-formation is attributed to ammonia excreted from the planarians (Kanatani, 1957b). Abnormal eye-formation in regenerating heads is observed in planarians treated with some drugs: p-chloromercurbenzoic acid (Smith and Hammen, 1963), lithium chloride (Brøndsted, 1942; Kanatani, 1958) and thyroxine (Mengebier, 1961). These studies of crowding and drug effects were performed to perturb the physiological states of planarians, and did not focus on the developmental aspects of eye formation.

Supernumerary eyes may inform us about the mechanisms normally used to organize eyes. The positions of supernumerary eyes may reveal how the field to regenerate eyes is formed after decapitation. As daily turnover of the photoreceptors occurs in planarians (Tamamaki, 1990), their eyes must be dynamically maintained. Are supernumerary eyes maintained dynamically and functionally? As supernumerary eyes are ectopic in a sense, it is of interest to know whether they retain regeneration ability or not. In this study, we collected a number of planarians with supernumerary eyes from

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our culture stock, classified the types of supernumerary eyes, and studied their regeneration ability. Furthermore, supernumerary eyes viewed as ectopic eyes cast light on the problem of how the optic nerves select the pathway towards the brain. This problem was approached by an immuno-histochemical study of the supernumerary eyes using MA-VC1.

MATERIALS AND METHODS

Animals

The planarians used in this study were a clonal population (GI-strain) derived from one *Dugesia japonica* animal collected from the Irima River, Gifu prefecture, Japan. They were fed chicken liver twice a week and cultured in autoclaved, cooled tap water at 22°C. Planarians with supernumerary eyes were collected from the culture stock. Before the operations, they were starved for at least 1 week. They were cold-anesthetized on ice, and their eyes were enucleated with fine glass capillaries.

Immunostaining

The planarians were soaked in 2-propanol chilled with dry ice and immediately transferred into a fixative of 10% formalin diluted with phosphate buffered saline (PBS). They were kept overnight at room temperature, dehydrated with a series of ethanol and 1-butanol, embedded in Paraplast (Oxford Lab.), and sectioned at 8 μm. After removing Paraplast, the sections were immunostained at room temperature. They were blocked with PBS containing 0.02% Triton X-100 (TPBS) for 10 min and washed twice with PBS. They were soaked in MA-VC1 (1000-fold diluted ascites) for 1 hour, and washed with TPBS and PBS. Then they were treated with 200-fold diluted FITC-labeled anti-mouse antibody (KPL Inc.) for 1 hr, washed and mounted in glycerol-PBS.

Assay of photophobic response

Planarians were dark-adapted at least one day before the assay of photophobic response. One planarian was transferred into a water pool (oval shape: 30 mm long axis and 15 mm short axis) on a whole slide glass, which were painted with black acryl paint to unreflect the light. The pool was irradiated (illuminance 100 lx) by a white light from the upper side. Three successive trials of 5 min each were forced to the planarian. On the first and second trials, left half side of the pool was shaded with a black paper, forming dark and light conditions in a pool. On the third trial, the black paper was removed. Duration of the planarian at the left side was recorded.

The statistical significance of the difference between samples was determined by *t* test. A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS

Types of supernumerary eyes

In our stock culture, planarians propagate asexually by fission. About one in 100 head regenerations is accompanied by formation of supernumerary eyes. Supernumerary eyes are formed due to a mistake during the regeneration process and not due to a genetic defect, because the planarians used in the present study are clonal. Furthermore, when planarians with supernumerary eyes are cut into anterior and posterior pieces, the posterior pieces regenerate heads with a pair of normal eyes (data not shown).

Supernumerary eyes of planarians were variable in position and morphology (Fig. 1). There were planarians with a supernumerary eye anterior or posterior to the normal pair of eyes. Planarians with four or five eyes were also found. In

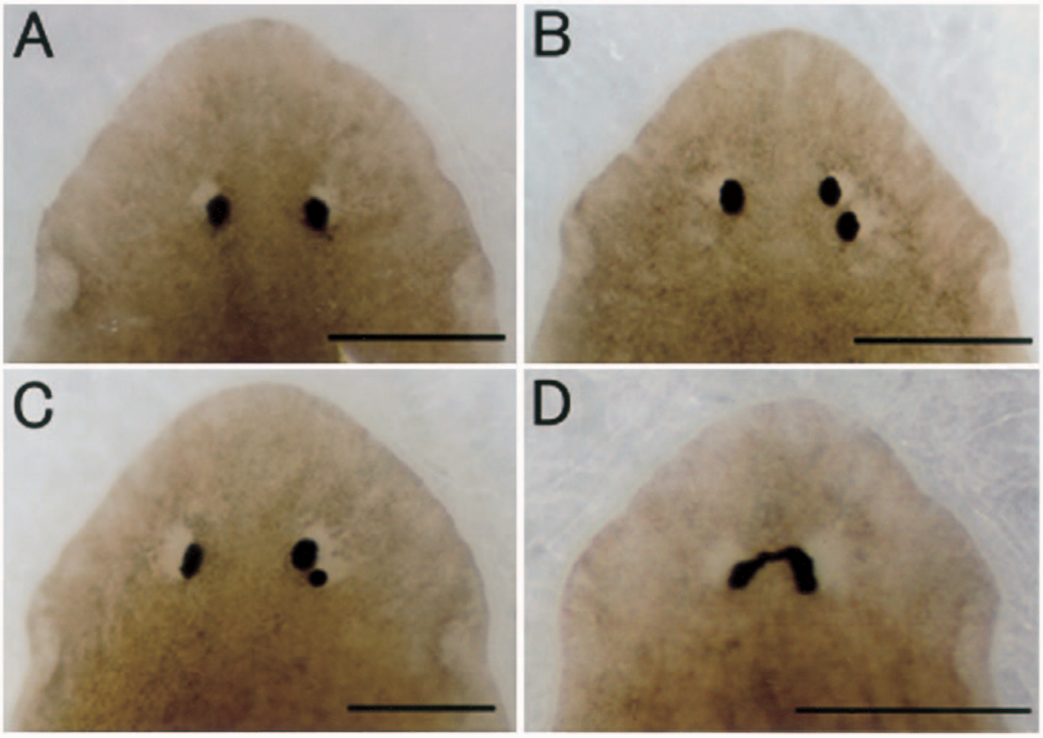

Fig.1. Planarians with normal (A) and supernumerary eyes (B-D). Two independent eyes (B) on the right side and partially fused eyes (C). (D) U-shaped eye. Bar 0.5 mm.

some cases, the eyes on the same side were independent. In other cases, the eyes were partially fused, that is, the eyes comprised a group of photoreceptors with two pigment eye cups. We found peculiar cases (about one in 3000 planarians) in which the right and left eyes were continuous, forming a U-shaped eye (Fig. 1D). Most of these U-shaped eyes were observed in young regenerants, and were disconnected within a few weeks, forming two normal eyes with or without supernumerary eyes. The other types of supernumerary eyes were stable for a year. Normal and supernumerary eyes were formed in the anterior part of the head.

Regeneration inability of the supernumerary eye

We examined whether the supernumerary eyes could regenerate or not. First, we used planarians with a supernumerary eye positioned posteriorly to the normal eye. Here, the two symmetrical eyes were designated as the normal eyes, and the other eye as a supernumerary. The eyes were removed in seven possible ways (Fig. 2), for each of which five planarians were used. In all cases, removal of the normal eyes elicited eye regeneration, but removal of supernumerary eyes did not. Next, the regeneration ability of a supernumerary eye positioned anteriorly to the normal eye was studied (Fig. 3).

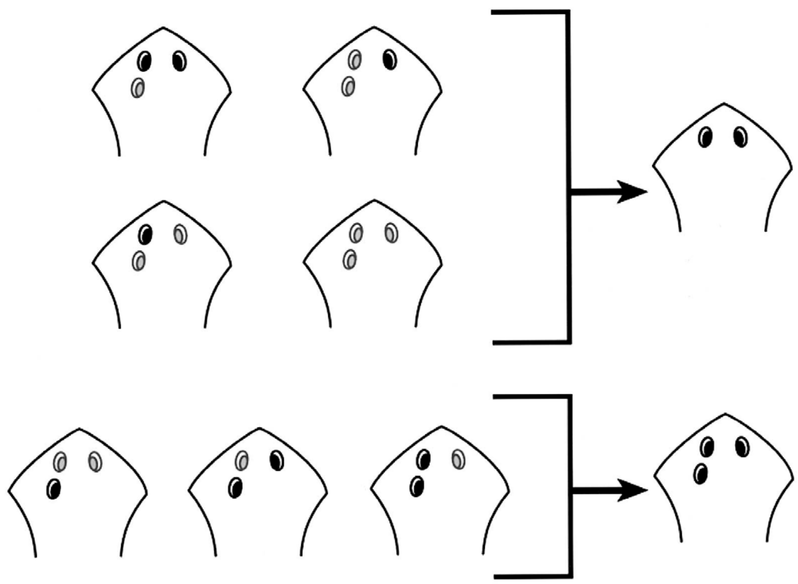

Fig. 2. Planarians with three eyes (a supernumerary eye situated posteriorly to the normal eye) were operated on in seven possible ways. The eyes drawn in gray represent removed eyes. In all cases, the normal eye could regenerate, and the supernumerary eye could not.

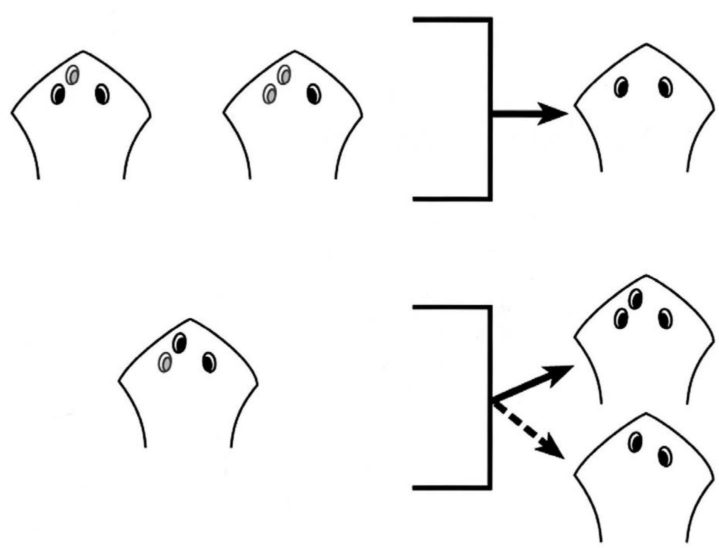

Fig. 3. Regeneration ability of a supernumerary eye positioned anteriorly to the normal eye. The eyes were removed in three ways from a unilateral site in planarians with a supernumerary eye. The eyes drawn in gray represent removed eyes. As a rule, the normal eyes regenerated, but supernumerary eyes did not. An exceptional case (one in five planarians operated on) has no regeneration of a normal eye after the single removal of one normal eye.

The eyes were removed in three ways from a unilateral site in planarians with a supernumerary eye. In this operation also, removal of the normal eyes elicited the eye regeneration as a rule, but removal of supernumerary eyes did not. An exceptional case (one among five planarians operated) had no eye regeneration after the removal of one normal eye (see Discussion).

Projection of optic nerves from the normal eye

The optic nerves from both eyes projected onto the brain, and formed a distinct optic chiasma (Fig. 4A, B). The optic nerve fibers innervated the dorso-medial area of the brain. A small number of optic nerve fibers extended toward the epidermis (Fig. 4C).

The optic chiasma might be partial, because optic nerves from the right eye projected onto both sides of the brain after the left eye was removed (Fig. 4D). Removal of both eyes abolished all of the immuno-reactive fibers (data not shown). Based on these results, the visual system of the planarian is represented schematically in Fig. 4E.

Projection of optic nerves from the supernumerary eyes

The projection patterns of optic nerves from the normal and supernumerary eyes were examined in order to determine their differences. In the planarians with three eyes, two independent eyes on the same side (11 cases, Figs. 1B) or

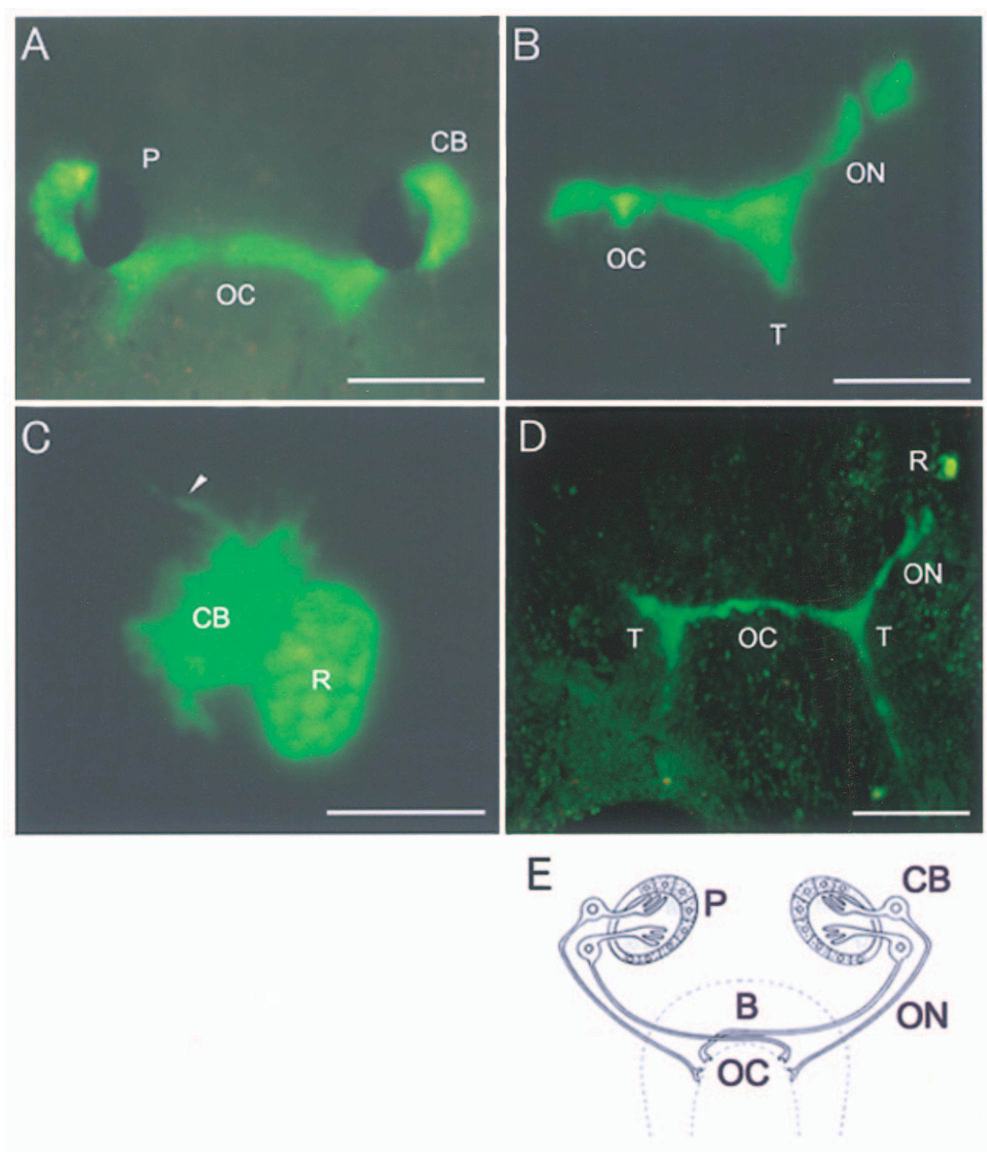

Fig. 4. Planarian photoreceptors stained with MA-VC1. The upper side is anterior and the lower side is posterior. (A) Whole-mount staining of a normal planarian. (B), (C) horizontal sections of normal planarians. Arrowhead in (C) indicates optic nerves extending towards the epidermis. (D) The day after removal of the left eye, no optic nerve fiber was observed around the area operated on, and the optic nerves from the right eye projected onto both sides of the brain. (E) Schema of the optic nerve pathway, assuming no branching of optic nerves. B, brain: CB, cell bodies of photoreceptor cells: OC, optic chiasma: ON, optic nerves: P, pigment eye cup: R, rhabdomeres in pigment eye cup: T, optic nerve terminals. Scale bars are 150 µm in A, B, and D and 50 µm in C.

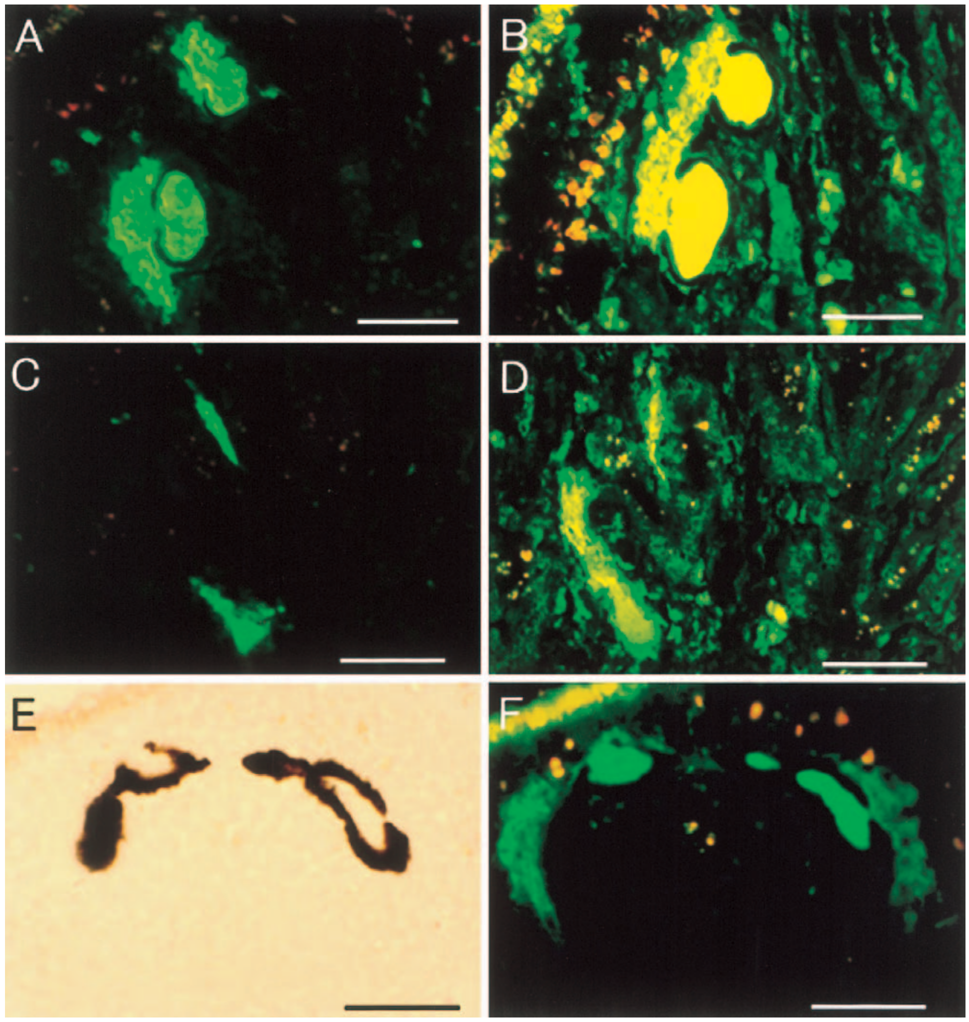

Fig. 5. Planarians with supernumerary eyes, shown by horizontal sections. (A, C) Sections of a planarian with independent eyes. (B, D) Sections of a planarian with partially fused eyes. Two groups of photoreceptors are present independently (A). One group of photoreceptors with two pigment eye cups are present in the partially fused eye (B). Individual optic nerve bundles exit from the independent eyes (C) and from the partially fused eyes (D). (E, F) A U-shaped eye. (E) Fused small pigment eye cups observed under transparent light. (F) Each pigment eye cup is associated with VC1 immuno-reactivity. The same field as in (E). Bar 100 µm.

partially fused eyes (7 cases, Figs. 1C) were examined. In a planarian with the independent eyes, two independent groups of photoreceptors (Fig. 5A) and the two optic nerve bundles (Fig. 5C) were observed. In a planarian with the partially fused eye, one group of photoreceptors with two pigment eye cups were observed (Fig. 5B). In this case also, two optic nerves ran independently (Figs. 5D). The optic nerve bundle of a fore eye always ran medially, and that of a hind eye ran laterally. They both projected onto the same area of the brain. The U-shaped eye (5 cases) was a battery of small pigment eye cups associated with photoreceptors (Fig. 5E, F). The supernumerary eye, including the projection pattern of the optic nerve, was not morphologically distinguishable from the normal eye.

Photophobic response of the planarian mediated by a supernumerary eye

In order to confirm whether the supernumerary eye mediates the light signal correctly to the brain, photophobic response of the planarian with a supernumerary eye was investigated. Normal eyes were removed from five planarians with a supernumerary eye positioned posteriorly to the normal eye. As the controls, 15 planarians with a normal pair of eyes were divided into three groups; both eyes were intact in one group, the right eye was enucleated in the second group, and both eyes were removed in the third group. The planarians were assayed two days after the operations.

When the intact planarian was put into a small pool on a whole slide glass under a white light, it swam around the pool. When half of the pool was shaded with a black paper, it tended to escape under the shadow. The planarian moved smoothly from the light sector to dark sector. However, when it transited from the dark sector to light sector, it turned the direction in many cases at the boundary of the dark and light sectors (photophobic response, Ullyott, 1936). As a result, duration of the intact planarian at the dark sector on the first and second

Table 1. Photohobic response after various operations

Sample	Intact		Removal of a right eye		Removal of both eyes		One supernumerary eye	
1	280	(169)	236	(169)	182	(139)	249	(153)
2	250	(177)	201	(155)	172	(148)	214	(137)
3	243	(151)	200	(155)	172	(148)	206	(149)
4	224	(161)	195	(152)	169	(163)	197	(158)
5	189	(168)	184	(140)	164	(153)	193	(160)
Mean	237.2*	(165.2)	203.2*	(154.2)	170.4	(154.2)	211.8*	(151.4)
SEM	15.1	(4.4)	8.7	(4.6)	3.2	(5.2)	10.0	(4.1)

The mean of duration (seconds) at the dark sector of the first and second trials, and duration at the half sector on the third trial (in parenthesis). The asterisks represent significant difference between experimental (first and seconnd trials) and control (third trial) groups.

trials was longer than its duration at the light sector (Table). When a shading black paper was removed on the third trial, its duration at the half site of a pool was about half of the trial time. The planarians with only a left eye sometimes missed the photophobic response, and so their duration at the dark sector is shorter than the duration of the intact animals. The planarians with no eye swam around the pool randomly. The planarians with only a supernumerary eye behaved similarly to the planarians with only a normal eye. The results of experimental (first and second) trials and control (third) trial were significantly different in case of planarians with eyes or an eye, and were not significantly different in case of planarians with no eye. Therefore, a supernumerary eye also mediates the light information correctly to the brain.

DISCUSSION

Eye regeneration field

There are various types of supernumerary eyes in planarians. It should be noted that normal and supernumerary eyes appear in a restricted area: the anterior part of the head. A similar situation is observed in other planarian species (genus *Polycelis*) which have a number of small oceli in the anterior part of the head (Aikawa and Shimozawa, 1991). The positions of the normal and supernumerary eyes may reflect the eye regeneration field postulated to exist in the anterior part of the regenerating head. We propose a model of eye regeneration field that is broad in the early phase of regeneration, and then usually becomes restricted into separate right and left areas (Fig. 6). Supernumerary eyes may be the outcome of mis-restriction of the eye regeneration field. Especially, restriction of the eye regeneration field may be delayed in the case of U-shaped eyes, which are observed in a small fraction of young regenerants. The eye regeneration field in planarians somewhat resembles the vertebrate eye primordium, which is situated at the anterior neural plate and then divided into right and left eye vesicles (Mangold, 1931).

Once formed, the supernumerary eyes are very stable, and are not distinguishable histologically from the normal eyes. Considering the daily turnover of the cells in planarian eyes (Tamamaki, 1990), new cells seem likely to be supplied to the supernumerary eyes as well as the normal eyes. Furthermore the supernumerary eye is as functional as the normal eye. Nevertheless, the supernumerary eyes and normal eyes are clearly different with respect to their regeneration ability. After removal of a normal eye, the planarian regenerates a new eye in the same region. The remaining supernumerary eye does not affect the regeneration of the normal eye. In contrast, the removal of a supernumerary eye does not elicit regeneration of a new eye, whether a normal eye is present or not. The tissues surrounding a supernumerary eye thus seem to lack the eye regeneration field. That is, the eye regeneration field is strictly restricted to the region of the normal eyes. No eye regeneration was found in one case of the operations in which a normal eye was removed and an anterior supernumerary eye was left intact. This may be attributable to the very closeness of the anterior supernumerary eye to the normal eye. Although it is not known where the precur-

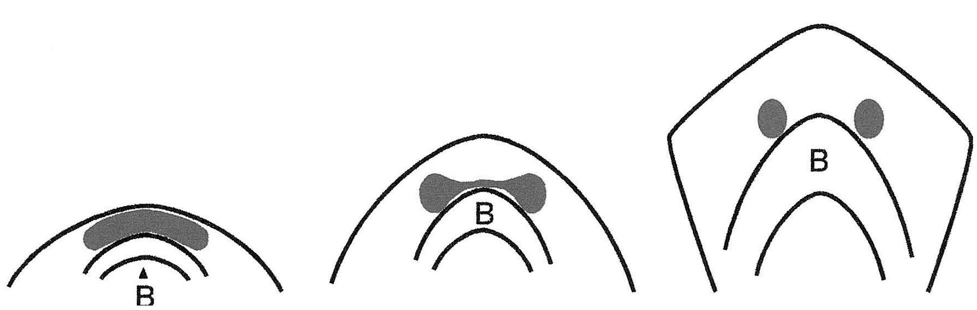

Fig. 6. Model for eye formation in regenerating head of planarians. The stages of regeneration proceed from left to right. The eye-forming or regeneration field (red) is broad in the anterior part of the head in the early phase of regeneration, and usually becomes restricted to two separate areas. B; brain.

sor cells of the eye are distributed, they do not differentiate at nor do they migrate to the position of enucleated supernumerary eyes. The molecular nature of the eye regeneration field remains to be determined.

Projection of the optic nerves

The planarian eye is composed of pigment cells and photoreceptors, from which the optic nerves extend towards the brain. The partial optic chiasma is the most remarkable feature in the projection pattern of optic nerves. Although planarians are simple bilateria, their partial optic chiasma enables them to integrate bilateral photo-sensory input at the visual center and to obtain differential information about the environment on both sides. The optic nerves definitely project onto the dorso-medial site of the brain. The stereo-projection pattern of the optic nerves in planarians suggests that several mechanisms are working to determine the pathway of optic nerves. In vertebrates, there are recognition mechanisms between the optic nerve terminals and the area of the brain onto which they project (Fujisawa *et al.*, 1989; Chen *et al.*, 1997) and a midline recognition mechanism at the optic chiasma (Wizenmann *et al.*, 1993; Kidd *et al.*, 1998).

In general, growth cones of neurons appear to be guided by at least four different mechanisms: contact attraction, chemoattraction, contact repulsion, and chemorepulsion (rev. Tessier-Lavigne and Goodman, 1996). What mechanisms guide the optic nerves to the brain in planarians? Two points are emphasized from the study of optic nerve pathways of planarians with a supernumerary eye. First, independent bundles of optic nerves extend from the normal and supernumerary eye cups. Second, the two optic nerve bundles run straight to the brain. It seems unlikely that there is a label on the pathway to guide the optic nerves in the planarian, because such a mechanism cannot explain the independence of the two optic nerve bundles. Rather, chemoattractants from the brain may guide the optic nerves.

Unexpectedly, we observed two optic nerve bundles from a group of photoreceptors with two pigment eye cups. Usually, nerve fibers with similar properties run together, due to the action of molecules such as fasciclin (Bastiani *et al.*, 1987; Grenningloh *et al.*, 1991). Some other mechanisms are required in order to explain the presence of the two optic nerve bundles. The interaction among rhabdomeres in an eye cup may be responsible for the formation of an optic nerve bundle. However, morphological or physiological experiments will be required to examine the interactions among rhabdomeres.

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